FORM PTO-1390 (REV 5-93) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U S APPLICATION NO (If known, see 37 CFR 1.5)
2139.22	09/787593

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP99/04128	30 July 1999	18 September 1998

TITLE OF INVENTION

METHOD FOR FRACTIONAL DETERMINATION OF CHOLESTEROL IN LIPOPROTEINS AND A REAGENT THEREFOR

APPLICANT(S) FOR DO/EO/US

Hiroyuki Sugiuchi

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the application time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:. Request for Approval of Proposed Drawing Changes; cover page of PCT application No. WO 00/17388; International search Report; Form PCT/IB301; FORM PCT/IB/304 and FORM PCT/IB/308.

U.S. APPLICATION NO (If known, see 37 CFR 1.5) 09/787393	INTERNATIONAL APPLICATION NO PCT/JP99/04128	ATTORNEY'S DOCKET NUMBER 2139.22

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5):		
Search Report has been prepared by the EP or JPO **$860.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.492(a)(1)) **$690.00**		
No international preliminary examination fee paid to USPTO (37 CFR 1.492 (a)(1)) but international search fee paid to USPTO (37 CFR 1.492(a)(2)) **$710.00**		
Neither international preliminary examination fee (37 CFR 1.492(a)(1)) nor international search fee (37 CFR 1.492(a)(2)) paid to USPTO **$1,000.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.492 (a)(4)) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$1,000.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	48 - 20 =	28	X **$18.00**	$504.00	
Independent Claims	8 - 3 =	5	X **$80.00**	$400.00	
Multiple dependent claim(s) (if applicable)			+ **$270.00**	$270.00	
TOTAL OF ABOVE CALCULATIONS =				$2,174.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$2,174.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$2,214.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$40.00	
TOTAL FEES ENCLOSED =				$	
				Amount to be:	
				refunded	$
				charged	$

a. ☒ A check in the amount of $ 2,214.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1205. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Jack M. Arnold
SIGNATURE

Jack M. Arnold
NAME

25,823
REGISTRATION NUMBER

2139.22

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	:	Examiner: Not Yet Assigned
HIROYUKI SUGIUCHI	)	
	:	Group Art Unit: N/Y/A
Application No.: N/Y/A	)	
	:	
Filed: Currently herewith	)	
	:	
For: METHOD FOR FRACTIONAL DETERMINATION OF CHOLESTEROL IN LIPOPROTEINS AND A REAGENT THEREFOR	)	
	:	
	)	March 16, 2001

Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Prior to action on the merits, please amend the above-identified application as follows:

IN THE SPECIFICATION:

Please substitute the paragraph starting at page 15, line 15 and ending at page 15, line 22 with the following replacement paragraph as follows. A marked-up copy of this section, showing the changes made thereto, is attached.

As the surfactant for activating the CH enzymes, anionic surfactants are used, e.g., at a concentration of 0 to

5%. Examples of the cholic acid are cholic acid, deoxycholic acid, taurocholic acid and chenodeoxycholic acid. The cholic acid is used at a concentration of 0 to 5%. Examples of the anionic surfactant include an alkyl sulfonate such as 1-pentasulfonate, 1-hexasulfonate, 1-heptasulfonate and 1-octasulfonate. These surfactants are used at a concentration of 0 to 5%.

Please substitute the paragraph starting at page 20, line 4 and ending at page 20, line 9 with the following replacement paragraph as follows. A marked-up copy of this section, showing the changes made thereto, is attached.

FIG. 3 is a graph showing the correlation between the concentration of total cholesterol obtained by the method of the present invention (designated by DB-TC in the figure) and the concentration of total cholesterol obtained by the comparative method (L TC II method, designated by L TC II in the figure).

Please substitute the paragraph starting at page 23, line 22 and ending at page 23, line 30 with the following replacement paragraph as follows. A marked-up copy of this section, showing the changes made thereto, is attached.

To determine HDL cholesterol and LDL cholesterol, substantially the same procedure as in Example 1 was repeated using the same samples as in Example 1 except that the wavelength

measured was changed to 555 nm. The coefficient of correlation between the results obtained with the commercial kits of Determiner L HDL-C and Determiner L LDL-C and the results obtained according to the method of the present invention was calculated. The coefficient of correlation showed 0.929 for the HDL cholesterol and 0.911 for the LDL cholesterol.

At page 24, line 8 please substitute the following replacement line as follows. A marked-up copy of this section, showing the changes made thereto, is attached.:

cholesterol oxidase (*2)

Please substitute the paragraph starting at page 24, line 19 and ending at page 24, line 21 with the following replacement paragraph as follows. A marked-up copy of this section, showing the changes made thereto, is attached.

Serum samples from 30 healthy subjects used in Example 1 were prepared and HDL cholesterol and [LDL] <u>total</u> cholesterol of the samples were determined by the following procedures.

Please substitute the paragraph starting at page 25, line 16 and ending at page 25, line 21 with the following replacement paragraph as follows. A marked-up copy of this section, showing the changes made thereto, is attached.

Fig. 3 shows a correlation between the concentration (mg/dL) of the total cholesterol according to the method of this

invention (designated as DB-TC in Fig. 3) and the concentration (mg/dL) of the total cholesterol obtained by the comparative method (L TC II method, designated as L TC II in Fig. 3).

Please substitute at page 27, Table 1, the following replacement Table as follows. A marked-up copy of this section, showing the changes made thereto, is attached.

Table 1

Surfactant	Concentration (%)	A_{HDL}/A_{LDL}	A_{VLDL}/A_{LDL}	A_{CM}/A_{LDL}
Pluronic L-121 Emulgen L-40	0.2 0.16	7.3	6.6	4.6
Pluronic L-121 Nonion HS-210	0.2 0.1	9.6	13.5	3.2
Pluronic L-121 Emulgen	0.2 0.1	10.2	7.7	1.2
Pluronic L-122 Emulgen L-40	0.2 0.16	8.1	8.2	3.4
Pluronic L-121 (comparative example 1)	0.2	34.7	47.9	16.8
Emulgen L-40 (comparative example 2)	0.16	27.8	39.7	9.7
Nonion HS-210 (comparative example 3)	0.1	35.5	35.5	6.1
Nonion HS-215 (comparative example 4)	0.16	76.8	33.6	4.7
Nonion NS-208.5 (comparative example 5)	0.24	44.5	32.4	51.2
Nonion HS-208 (comparative example 6)	0.08	30.2	47.3	28.3

Surfactant	Concentration (%)	A_{HDL}/A_{LDL}	A_{VLDL}/A_{LDL}	A_{CM}/A_{LDL}
Emulgen 911 (comparative example 7)	0.1	22.6	15.9	3.0
Emulgen 810 (comparative example 8)	0.2	24.7	36.8	5.8
Pluronic L-122 (comparative example 9)	0.2	38.1	64.1	19.0

IN THE CLAIMS:

Please amend Claims 9, 12, 14, 16 and 27 as follows. A marked up version of the amended claims is attached.

9. (Amended) The method according to claim (7), wherein the polyoxyethylene-polyoxypropylene copolymer is a surfactant represented by general formula (I):

$$HO-(C_2H_4O)_a-(C_3H_6O)_b-(C_2H_4O)_c-H \qquad (I)$$

(wherein a, b and c, which may be the same or different, each represents an integer of 1 to 200).

12. (Amended) The method according to any one of claims (5), (6), (10) or (11), wherein the reagent enabling CH enzymes to act only on cholesterol in HDL is a reagent for aggregating lipoproteins other than HDL.

14. (Amended) The method according to claim (12), wherein the reagent for aggregating lipoproteins other than HDL is a reagent comprising heparin or a salt thereof, phosphotungstic acid or a salt thereof, dextran sulfuric acid or a salt thereof, polyethylene glycol, sulfonated cyclodextrin or a salt thereof, sulfonated oligosaccharide or a salt thereof, or a mixture thereof and a divalent metal salt.

16. (Amended) The method according to any one of claims (10) or (11), wherein the reagent enabling the CH enzymes to act on cholesterol in all lipoproteins is a reagent containing a lipoprotein solubilizing surfactant.

27. (Amended) The reagent kit according to any one of claims (21), (22), (23), (25) or (26), wherein the reagent for aggregating lipoproteins other than HDL lipoprotein is a reagent comprising heparin or a salt thereof, phosphotungstic acid or a salt thereof, dextran sulfuric acid or a salt thereof, polyethylene glycol, sulfonated cyclodextrin or a salt thereof, sulfonated oligosaccharide or a salt thereof, or a mixture thereof and a divalent metal salt.

REMARKS

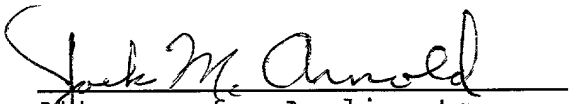
The specification has been amended to correct some typographical errors. Claims 9, 12, 14, 16 and 27 have been amended to correct their dependency and conformity with accepted U.S. practice.

No new matter has been added.

Entry hereof is earnestly solicited.

Applicants' undersigned attorney may be reached in our New York office by telephone at (212) 218-2100. All correspondence should continue to be directed to our below listed address.

Respectfully submitted,



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NY_MAIN 154448 v1

Application No.: Not Yet Assigned
Attorney Docket No.: 2139.22

VERSION WITH MARKINGS TO SHOW CHANGES MADE TO SPECIFICATION

The paragraph starting at page 15, line 15 and ending at page 15, line 22 has been amended as follows:

As the surfactant for activating the CH enzymes, anionic surfactants are used, e.g., at a concentration of 0 to [1]5%. Examples of the cholic acid are cholic acid, deoxycholic acid, taurocholic acid and chenodeoxycholic acid. The cholic acid is used at a concentration of 0 to 5%. Examples of the anionic surfactant include an alkyl sulfonate such as 1-pentasulfonate, 1-hexasulfonate, 1-heptasulfonate and 1-octasulfonate. These surfactants are used at a concentration of 0 to 5%.

The paragraph starting at page 20, line 4 and ending at page 20, line 9 has been amended as follows:

FIG. 3 is a graph showing the correlation between the concentration of total cholesterol obtained by the method of the present invention (designated by DB-TC in the figure) and the concentration of total cholesterol obtained by the comparative method ([Determiner] L TC II method, designated by L TC II in the

figure).

The paragraph starting at page 23, line 22 and ending at page 23, line 30 has been amended as follows:

To determine HDL cholesterol and LDL cholesterol, substantially the same procedure as in Example 1 was repeated using the same samples as in Example 1 except that the wavelength measured was changed to 555 nm. The coefficient of correlation between the results obtained with the commercial kits of Determiner L HDL-C and Determiner L [HDL-C] LDL-C and the results obtained according to the method of the present invention was calculated. The coefficient of correlation showed 0.929 for the HDL cholesterol and 0.911 for the LDL cholesterol.

Page 24, line 8 has been amended as follows:

cholesterol oxidase (*[1]2)

The paragraph starting at page 24, line 19 and ending at page 24, line 21 has been amended as follows:

Serum samples from 30 healthy subjects used in Example 1 were prepared and HDL cholesterol and [LDL] total cholesterol of the samples were determined by the following procedures.

The paragraph starting at page 25, line 16 and ending at page 25, line 21 has been amended as follows:

Fig. 3 shows a correlation between the concentration (mg/dL) of the total cholesterol according to the method of this invention (designated as DB-TC in Fig. 3) and the concentration (mg/dL) of the total cholesterol obtained by the comparative method ([Determiner] L TC II method, designated as L TC II in Fig. 3).

The table at page 27 has been amended as follows:

Table 1

Surfactant	Concentration (%)	A_{HDL}/A_{LDL}	A_{VLDL}/A_{LDL}	A_{CM}/A_{LDL}
Pluronic L-121 Emulgen L-40	0.2 0.16	7.3	6.6	4.6
Pluronic L-121 Nonion HS-210	0.2 0.1	9.6	13.5	3.2
Pluronic L-121 Emulgen	0.2 0.1	10.2	7.7	1.2
Pluronic L-122 Emulgen L-40	0.2 0.16	8.1	8.2	3.4
Pluronic L-121 (comparative example 1)	0.2	34.7	47.9	16.8
Emulgen L-40 (comparative example 2)	0.16	27.8	39.7	9.7
Nonion HS-210 (comparative example 3)	0.1	35.5	35.5	6.1

Surfactant	Concentration (%)	A_{HDL}/A_{LDL}	A_{VLDL}/A_{LDL}	A_{CM}/A_{LDL}
Nonion HS-215 (comparative example 4)	0.16	76.8	33.6	4.7
Nonion NS-208.5 (comparative example 5)	0.24	44.5	32.4	51.2
Nonion [NS-208] HS-208 (comparative example 6)	0.08	30.2	47.3	28.3
Emulgen 911 (comparative example 7)	0.1	22.6	15.9	3.0
Emulgen 810 (comparative example 8)	0.2	24.7	36.8	5.8
Pluronic L-122 (comparative example 9)	0.2	38.1	64.1	19.0

NY_MAIN 154448 v1

VERSION WITH MARKINGS TO SHOW CHANGES MADE TO CLAIMS

9. (Amended) The method according to claim (7) [or (8)], wherein the polyoxyethylene-polyoxypropylene copolymer is a surfactant represented by general formula (I):

$$HO-(C_2H_4O)_a-(C_3H_6O)_b-(C_2H_4O)_c-H \quad (I)$$

(wherein a, b and c, which may be the same or different, each represents an integer of 1 to 200).

12. (Amended) The method according to any one of claims (5), [through] (6), (10) or (11), wherein the reagent enabling CH enzymes to act only on cholesterol in HDL is a reagent for aggregating lipoproteins other than HDL.

14. (Amended) The method according to claim (12) [or (13)], wherein the reagent for aggregating lipoproteins other than HDL is a reagent comprising heparin or a salt thereof, phosphotungstic acid or a salt thereof, dextran sulfuric acid or a salt thereof, polyethylene glycol, sulfonated cyclodextrin or a salt thereof, sulfonated oligosaccharide or a salt thereof, or a mixture thereof and a divalent metal salt.

16. (Amended) The method according to any one of claims (10) [through (15)] or (11), wherein the reagent enabling the CH enzymes to act on cholesterol in all lipoproteins is a reagent containing a lipoprotein solubilizing surfactant.

27. (Amended) The reagent kit according to any one of claims (21), [through] (22), (23), (25) or (26), wherein the reagent for aggregating lipoproteins other than HDL lipoprotein is a reagent comprising heparin or a salt thereof, phosphotungstic acid or a salt thereof, dextran sulfuric acid or a salt thereof, polyethylene glycol, sulfonated cyclodextrin or a salt thereof, sulfonated oligosaccharide or a salt thereof, or a mixture thereof and a divalent metal salt.

NY_MAIN 154448 v 1

SPECIFICATION
METHODS FOR FRACTIONAL DETERMINATION OF CHOLESTEROL IN LIPOPROTEINS AND A REAGENT THEREFOR

Technical Field

The present invention relates to a method for the quantitative determination of cholesterol in low density lipoproteins (LDL) (hereinafter referred to as LDL cholesterol), which is important in the field of clinical diagnosis, and a reagent for use in the method. The present invention also relates to a method for the continuous fractional determination of cholesterol in high density lipoproteins (HDL) (hereinafter referred to as HDL cholesterol) and LDL cholesterol, which are also important in the field of clinical diagnosis, and a reagent kit for use therein. The present invention further relates to a method for the continuous fractional determination of HDL cholesterol, LDL cholesterol and total cholesterol [the term is used to mean total cholesterol in HDL, LDL, very low density lipoproteins (hereinafter referred to as VLDL) and chylomicron (hereinafter referred to as CM)], which are important in the field of clinical diagnosis, as well as a reagent kit to be used therefor.

Background Art

In general, HDL is called good cholesterol since HDL functions to remove cholesterol accumulated on arterial walls and transport cholesterol to liver. On the other hand, LDL is generally termed bad cholesterol because of its action to transport cholesterol to peripheral tissues including arterial walls. In the field of clinical investigations, the levels of HDL cholesterol, LDL cholesterol and total cholesterol are useful indices for total judgement of lipid-related diseases such as arteriosclerosis, etc.

These cholesterol levels are separately determined using reagents exclusively specific to each type of cholesterol so that an autoanalyzer is designed so as to be suitable for

individual determination of these cholesterol levels. It has been desired to further improve the specificity of a reagent to each cholesterol. Besides, no simple and automated method for the continuous fractional determination of HDL cholesterol, LDL cholesterol, total cholesterol, etc in the same detection system is known.

Disclosure of the Invention

An object of the present invention is to provide a method for the quantitative determination of LDL cholesterol and a determination reagent for use in such a method.

Another object of the present invention is to provide a method for the continuous fractional determination of HDL cholesterol and LDL cholesterol in the same sample and a reagent kit for use therein.

More specifically, the present invention relates to (1) through (27) below.

(1) A method for quantitatively determining LDL cholesterol in a biological sample, which comprises

performing the reaction of cholesterol in the presence of:

a) a biological sample,

b) cholesterol esterase and cholesterol oxidase or cholesterol dehydrogenase (hereinafter collectively referred to as CH enzymes), and

c) a reagent enabling the CH enzymes of b) to act only on LDL cholesterol, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the reaction to quantitatively determine the concentration of LDL cholesterol.

(2) The method according to (1), wherein the reagent enabling CH enzymes to act only on LDL cholesterol is a reagent containing at least a polyoxyethylene derivative and a polyoxyethylene-polyoxypropylene copolymer.

(3) The method according to (2), wherein the polyoxyethylene derivative is a polyoxyethylene alkyl ether or a polyoxyethylene alkylaryl ether.

(4) The method according to (2) or (3), wherein the polyoxyethylene-polyoxypropylene copolymer is a surfactant represented by general formula (I):

$$HO-(C_2H_4O)_a-(C_3H_6O)_b-(C_2H_4O)_c-H \qquad (I)$$

(wherein a, b and c, which may be the same or different, each represents an integer of 1 to 200).

(5) A method for the continuous fractional determination of HDL cholesterol and LDL cholesterol in a biological sample, which comprises

subjecting cholesterol to the first reaction in the presence of:

a) a biological sample,

b) CH enzymes, and

c) a reagent enabling the CH enzymes of b) to act only on HDL cholesterol, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the first reaction to quantitatively determine the concentration of HDL cholesterol,

then adding

d) a reagent enabling the CH enzymes of b) to act only on LDL cholesterol,

subjecting cholesterol to the second reaction, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the second reaction to quantitatively determine the concentration of LDL cholesterol.

(6) A method for the continuous fractional determination of HDL cholesterol and LDL cholesterol in a biological sample, which comprises

subjecting cholesterol to the first reaction in the presence of:

a) a biological sample,

b) CH enzymes, and

c) a reagent enabling the CH enzymes of b) to act only on HDL cholesterol, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the first reaction to quantitatively determine the concentration of HDL cholesterol,

then adding

d) CH enzymes, and

e) a reagent enabling the CH enzymes of d) to act only on LDL cholesterol,

subjecting cholesterol to the second reaction, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the second reaction to quantitatively determine the concentration of LDL cholesterol.

(7) The method according to (5) or (6), wherein the reagent enabling CH enzymes to act only on LDL cholesterol is a reagent containing at least a polyoxyethylene derivative and a polyoxyethylene-polyoxypropylene copolymer.

(8) The method according to (7), wherein the polyoxyethylene derivative is a polyoxyethylene alkyl ether or a polyoxyethylene alkylaryl ether.

(9) The method according to (7) or (8), wherein the polyoxyethylene-polyoxypropylene copolymer is a surfactant represented by general formula (I):

$$HO-(C_2H_4O)_a-(C_3H_6O)_b-(C_2H_4O)_c-H \qquad (I)$$

(wherein a, b and c, which may be the same or different, each represents an integer of 1 to 200).

(10) A method for the continuous fractional determination of HDL cholesterol and total cholesterol in a biological sample, which comprises

subjecting cholesterol to the first reaction in the presence of:

a) a biological sample,

b) CH enzymes, and

c) a reagent enabling CH enzymes of b) to act only on HDL cholesterol, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the first reaction to quantitatively determine the concentration of HDL cholesterol,

then adding

d) a reagent enabling the CH enzymes of b) to act on cholesterol in all lipoproteins,

subjecting cholesterol to the second reaction, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the second reaction to quantitatively determine the concentration of total cholesterol.

(11) A method for the continuous fractional determination of HDL cholesterol and total cholesterol in a biological sample, which comprises

subjecting cholesterol to the first reaction in the presence of:

a) a biological sample,

b) CH enzymes, and

c) a reagent enabling the CH enzymes of b) to act only on HDL cholesterol, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the first reaction to quantitatively determine the concentration of HDL cholesterol,

then adding

d) CH enzymes, and

e) a reagent enabling the CH enzymes of d) to act on cholesterol in all lipoproteins,

subjecting cholesterol to the second reaction, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the second reaction to quantitatively determine the total cholesterol.

(12) The method according to any one of (5) through (11), wherein the reagent enabling CH enzymes to act only on cholesterol in HDL is a reagent for aggregating lipoproteins other than HDL.

(13) The method according to (12), wherein the reagent for aggregating lipoproteins other than HDL further contains

a nonionic surfactant that does not solubilize the aggregated lipoproteins.

(14) The method according to (12) or (13), wherein the reagent for aggregating lipoproteins other than HDL is a reagent comprising heparin or a salt thereof, phosphotungstic acid or a salt thereof, dextran sulfuric acid or a salt thereof, polyethylene glycol, sulfonated cyclodextrin or a salt thereof, sulfonated oligosaccharide or a salt thereof, or a mixture thereof and a divalent metal salt.

(15) The method according to (6) or (11), wherein the CH enzymes used in the first reaction of cholesterol are chemically modified enzymes and the CH enzymes used in the second reaction of cholesterol are enzymes that are not chemically modified.

(16) The method according to any one of (10) through (15), wherein the reagent enabling the CH enzymes to act on cholesterol in all lipoproteins is a reagent containing a lipoprotein solubilizing surfactant.

(17) A reagent for determining LDL cholesterol comprising CH enzymes and a reagent enabling the CH enzymes to act only on LDL cholesterol.

(18) The reagent for determining LDL cholesterol according to (17), wherein the reagent enabling the CH enzymes to act only on LDL cholesterol is a reagent containing at least a polyoxyethylene derivative and a polyoxyethylene-polyoxypropylene copolymer.

(19) The reagent according to (18), wherein the polyoxyethylene derivative is a polyoxyethylene alkylaryl ether.

(20) The reagent according to (18) or (19), wherein the polyoxyethylene-polyoxypropylene copolymer is a surfactant represented by general formula (I):

$$HO-(C_2H_4O)_a-(C_3H_6O)_b-(C_2H_4O)_c-H \qquad (I)$$

(wherein a, b and c, which may be the same or different, each represents an integer of 1 to 200).

(21) A reagent kit for the fractional determination of HDL cholesterol and LDL cholesterol comprising a first reagent and a second reagent, said first reagent comprising a reagent for aggregating lipoproteins other than HDL lipoprotein and a reagent containing CH enzymes, and said second reagent comprising a reagent enabling CH enzymes to act only on LDL cholesterol.

(22) The reagent kit according to (21), wherein the reagent enabling CH enzymes to act only on LDL cholesterol is a reagent containing a polyoxyethylene derivative and a polyoxyethylene-polyoxypropylene copolymer.

(23) The reagent kit according to (21), wherein the polyoxyethylene derivative is a polyoxyethylene alkylaryl ether.

(24) The reagent kit according to (21) or (22), wherein the polyoxyethylene-polyoxypropylene copolymer is a surfactant represented by general formula (I):

$$HO-(C_2H_4O)_a-(C_3H_6O)_b-(C_2H_4O)_c-H \qquad (I)$$

(wherein a, b and c, which may be the same or different, each represents an integer of 1 to 200).

(25) A reagent kit for the fractional determination of HDL cholesterol and total cholesterol comprising a first reagent and a second reagent, said first reagent comprising a reagent for aggregating lipoproteins other than HDL lipoprotein and a reagent containing CH enzymes, and said second reagent comprising a reagent enabling CH enzymes to act on cholesterol in all lipoproteins.

(26) The reagent kit according to (25), wherein the reagent enabling CH enzymes to act on cholesterol in all lipoproteins further contains a lipoprotein solubilizing surfactant.

(27) The reagent kit according to any one of (21) through (26), wherein the reagent for aggregating lipoproteins other than HDL lipoprotein is a reagent comprising heparin or a salt thereof, phosphotungstic acid or a salt thereof, dextran sulfuric acid or a salt thereof, polyethylene glycol,

sulfonated cyclodextrin or a salt thereof, sulfonated oligosaccharide or a salt thereof, or a mixture thereof and a divalent metal salt.

Hereinafter the present invention will be described in more detail.

As described above, the present invention relates to a method for the quantitative determination of LDL cholesterol which comprises adding to a biological sample containing various types of lipoproteins a specific reagent enabling CH enzymes to act only on LDL cholesterol (hereinafter referred to as reagent A) and a reagent for use in such a method.

As described above, the present invention also relates to a method for the fractional determination of HDL cholesterol and LDL cholesterol which comprises adding to a biological sample containing various types of lipoproteins a specific reagent enabling CH enzymes to act only on HDL cholesterol (hereinafter referred to as reagent B) to quantitatively determine HDL cholesterol and then adding reagent A to quantitatively determine LDL cholesterol as well as a reagent kit for use therein.

As described above, the present invention further relates to a method for the fractional determination of HDL cholesterol and total cholesterol which comprises adding to a biological sample containing various types of lipoproteins the reagent B to quantitatively determine HDL cholesterol and then adding to the reaction mixture a reagent enabling CH enzymes to act on cholesterol in all lipoproteins (hereinafter referred to as reagent C) to quantitatively determine total cholesterol as well as a reagent kit for use therein.

A biological sample to which the present invention is to be applied is not particularly limited. More specifically, blood itself or blood fractions such as plasma or serum, etc. may be used as the sample.

The reactions for quantitatively determining cholesterol in the present invention are generally carried out in an aqueous medium, preferably in a buffer solution.

Buffers useful in the buffer solution include tris(hydroxymethyl)aminomethane, phosphate buffer, borate buffer and Good's buffer. Examples of Good's buffer are N-(2-acetamido)-2-aminoethanesulfonic acid (ACES), N-(2-acetamido)iminodiacetic acid (ADA), N, N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 3-[N,N-bis(2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid (DIPSO), N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 2-(N-morpholino)ethanesulfonci acid (hereinafter referred to as MES), 3-(N-morpholino)propanesulfonic acid (hereinafter referred to as MOPS), 3-(N-morpholino)-2-hydroxypropanesulfonic acid (MOPSO), piperazine-N,N'-bis(2-ethanesulfonic acid) (hereinafter referred to as PIPES), piperazine-N,N'-bis(2-hydroxypropane-3-sulfonic acid) (POPSO), etc.

The pH of the buffer solution is 5 to 10, preferably 6 to 9. The concentration of the buffer to be used is 5 to 500 mM, preferably 20 to 200 mM.

The reagent A which enables CH enzymes to act only on LDL cholesterol is a reagent that does not enable CH enzymes to act on cholesterol in HDL, VLDL and CM. The reagent A also enables CH enzymes to act only on LDL cholesterol even in the presence of a reagent for aggregating lipoproteins other than HDL, which will be later described.

The reagent A is typically a reagent containing at least a polyoxyethylene derivative and a polyoxyethylene-polyoxypropylene copolymer.

Suitable polyoxyethylene derivative is exemplified by a polyoxyethylene alkylaryl ether, a polyoxyethylene alkyl ether, etc., having the alkyl moiety of at least 8 carbon atoms, e.g., octyl, nonyl, etc. and having the aryl moiety being phenyl, etc.

Specific examples of the polyoxyethylene derivative include commercially available Nonion HS-210, Nonion HS-215,

Nonion NS-208.5 and Nonion HS-208 (all produced by NOF Corporation) and Emulgen L-40, Emulgen 911 and Emulgen 810 (all produced by Kao Corporation). The hydrophile-lipophile balance (hereinafter referred to as HLB) of the polyoxyethylene derivative is preferably 9 to 20.

The polyoxyethylene-polyoxypropylene copolymers may be either random copolymers or block copolymers of polyoxyethylene and polyoxypropylene. An example of the copolymer is a compound represented by general formula (I):

$$HO-(C_2H_4O)_a-(C_3H_6O)_b-(C_2H_4O)_c-H \qquad (I)$$

(wherein a, b and c, which may be the same or different, each represents an integer of 1 to 200).

Examples of the compounds represented by general formula (I) include commercially available compounds such as Pluronic L-121, Pluronic L-122, Pluronic L-101, Pluronic P-103 and Pluronic F-108 (all produced by Asahi Denka Kogyo K. K.). The molecular weight of the polypropylene glycol moiety in the compounds of general formula (I) is preferably at least 2,050, more preferably 2,750 or more, most preferably 3,250 or more. The HLB of the polyoxyethylene-polyoxypropylene copolymers is preferably 1 to 6.

The respective concentration of the polyoxyethylene derivatives and polyoxyethylene-polyoxypropylene copolymers used is not specifically limited but is preferably 0.001 to 10%, more preferably 0.01 to 5%, most preferably 0.05 to 1%.

Examples of the reagent B that enables CH enzymes to act only on HDL cholesterol are reagents for aggregating lipoproteins other than HDL and antibodies to lipoproteins other than HDL.

Reagents for aggregating lipoproteins other than HDL are generally those containing agents for aggregating these lipoproteins and/or divalent metal salts. Examples of the aggregating agent include heparin or salts thereof, phosphotungstic acid or salts thereof, dextran sulfuric acid or salts thereof, polyethylene glycol, sulfated cyclodextrin or salts thereof, sulfated oligosaccharide or salts thereof,

and mixtures thereof. Examples of the cyclodextrin include α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin. Examples of the oligosaccharide include maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. Examples of the salts include sodium, potassium, lithium, ammonium and magnesium salts. Examples of the divalent metal salt include magnesium, calcium, manganese and nickel salts.

Preferable examples of the aggregating agent used include 0.02 to 10 mM heparin having a molecular weight of 5,000 to 20,000 or salts thereof, 0.1 to 10 mM phosphotungstic acid having a molecular weight of 4,000 to 8,000 or salts thereof, 0.01 to 5 mM dextran sulfuric acid having a molecular weight of 10,000 to 500,000 or salts thereof, 0.1 to 20 mM dextran sulfuric acid having a molecular weight of 1,000 to 10,000 or salts thereof, 0.3 to 100 mM polyethylene glycol (PEG) having a molecular weight of 4,000 to 25,000, 0.1 to 50 mM sulfated cyclodextrin having a molecular weight of 1,000 to 3,000 or salts thereof, 0.1 to 50 mM sulfated oligosaccharide having a molecular weight of 400 to 3,000 or salts thereof, and mixtures thereof. More preferred examples are 0.03 to 1 mM heparin having a molecular weight of 14,000 to 16,000 or salts thereof, 0.1 to 3 mM phosphotungstic acid having a molecular weight of 5,000 to 7,000 or salts thereof, 0.01 to 5 mM dextran sulfuric acid having a molecular weight of 150,000 to 250,000 or salts thereof, 0.1 to 10 mM dextran sulfuric acid having a molecular weight of 1,000 to 5,000 or salts thereof, 1.0 to 50 mM PEG having a molecular weight of 5,000 to 22,000, 0.1 to 10 mM sulfated cyclodextrin having a molecular weight of 1,000 to 2,000 or salts thereof, 0.1 to 10 mM sulfated oligosaccharide having a molecular weight of 400 to 2,000 or salts thereof, and mixtures thereof.

Preferred examples of the divalent metal salt include the salts of magnesium, calcium, manganese, nickel and cobalt, the concentration of which is 0.1 to 50 mM. Preferably, the magnesium salt is used in a concentration of 0.1 to 50 mM.

It is preferred that the agents for aggregating lipoproteins other than HDL further contain a nonionic surfactant that does not dissolve the aggregated lipoproteins.

Examples of the nonionic surfactant that does not dissolve the aggregated lipoproteins include a polyoxyethylene alkyl ether, a polyoxyethylene alkylaryl ether, a polyoxyethylene-polyoxypropylene copolymer, a polyoxyethylene alkyl ether sulfuric acid salts and an alkylbenzene sulfonate. Among these surfactants, polyoxyethylene ethers [Emulgen 220 (Kao Corporation), etc.] are particularly desired as the polyoxyethylene alkyl ether; commercially available Emulgen 66, etc. as the polyoxyethylene alkyl aryl ether; commercially available Pluronic F88 (Asahi Denka Kogyo K. K.) as the polyoxyethylene-polyoxypropylene condensate, commercially available Emal 20C (Kao Corporation) as the polyoxyethylene alkyl ether sodium sulfate, and sodium dodecyl benzenesulfonate as the alkyl benzenesulfonic acid salt.

The nonionic surfactant that does not dissolve the aggregated lipoproteins can be used in combination, so long as the surfactant does not enable CH enzymes to act on LDL cholesterol. However, it is preferable to use the nonionic surfactant solely. The concentration of the nonionic surfactant is not particularly limited but is preferably 0.01 to 10%, more preferably 0.1 to 5%.

Examples of the antibodies to lipoproteins other than HDL include an antiapo-lipoprotein B antibody, an antiapo-lipoprotein C antibody, an antiapo-lipoprotein E antibody and an anti-β-lipoprotein antibody. These antibodies may be employed solely or in combination. The antibodies may be either polyclonal or monoclonal. The antibodies may also be chemically or enzymatically degraded or modified.

As the reagent C enabling CH enzymes to act on cholesterol in all lipoproteins, there are, for example, surfactants that dissolve all lipoproteins.

As the surfactants above, there are used nonionic surfactants that dissolve HDL, LDL, VLDL and CM. Specific examples of such surfactants are nonionic surfactants commercially available as Triton X-100, polyoxyethylene alkyl ethers such as Emulgen 106, Emulgen 108, Emulgen 709, etc. These surfactants may be used solely or in combination. The concentration of the surfactants is not particularly limited but is preferably 0.01 to 10%, more preferably 0.1 to 5%.

As the enzymes having the activities of cholesterol esterase, cholesterol oxidase and cholesterol dehydrogenase which may be used in the present invention, there are, for example, cholesterol esterase and lipoprotein lipase derived from microorganisms or animals having the ability to hydrolyze cholesterol ester, cholesterol oxidase derived from microorganisms having the ability to oxidize cholesterol to produce hydrogen peroxide, and cholesterol dehydrogenase derived from microorganisms or animals.

These enzymes can be employed depending upon specificity to substrate. In the case of the quantitative determination of HDL cholesterol, it is preferred to use an enzyme specific to the cholesterol and for the quantitative determination of LDL cholesterol, an enzyme specific thereto is preferably used. In order to further improve the specificity and stability of these enzymes, enzymes that are chemically modified with a group having polyethylene glycol as a main component, a water-soluble oligosaccharide residue, or a sulfopropyl group may also be used. Furthermore, enzymes obtained by genetic engineering may also be used.

Examples of the reagent for modifying the enzymes (chemical modifier) include compounds wherein polyethylene glycol and a group capable of bonding to an amino group are connected, e.g. Sun Bright VFM4101 (NOF Corporation) wherein polyethylene glycol and a group capable of bonding to an amino group such as N-hydroxysuccinimido group are connected, Sun Bright AKM series, ADM series and ACM series [all manufactured by NOF Corporation, Chemical Engineering Monographs (Kagaku

Kogaku Ronbunshu), 20 (3), 459 (1994)] which are compounds having the polyalkylene glycol structure and the acid anhydride structure, compounds wherein a polyethylene glycol-polypropylene glycol copolymer and a group capable of bonding to an amino group are connected, copolymers of polyethylene glycol monomethacryl monomethyl ether and maleic anhydride, etc. Furthermore, activated polyurethane P4000 (Boehringer Mannheim, Directions for Enzyme Modification Set) which is a polyurethane chemical modifier, Dextran T40, which is a dextran chemical modifier, and activated TCT (Boehringer Mannheim, Directions for Enzyme Modification Set), 1,3-propanesultone, etc. may also be used. By the use of these chemical modifiers, the enzymes can be modified with a group having polyethylene glycol as a main component, a group having polypropylene glycol as a main component, a group having a copolymer of polypropylene glycol and polyethylene glycol, a group containing a saccharide in the structure, a sulfopropyl group, a polyurethane group, etc.

A method for the reaction of an enzyme with the above chemical modifier is described in Yuji Inada, "Tanpakushitu-no-Hybrid (Hybrid of Proteins)" published by Kyoritsu Publishing Co. (1987), etc. Typically, when using, e.g., Sun Bright, the enzyme is dissolved in a buffer solution such as HEPES buffer of pH 8 or above, then, e.g., 0.01-500-fold molar amount of Sun Bright is added to the solution at 0℃ to 50℃, followed by stirring for 5 to 60 minutes. The resulting reaction mixture is used as it is, or if necessary, after removal of low molecular weight compounds with ultrafilter.

According to the present invention, the cholesterol esterase, cholesterol oxidase and cholesterol dehydrogenase are preferably used in the reaction mixture at a concentration of 0.01 to 200 U/ml, more preferably 0.1-100 U/ml.

In the present invention, the CH enzymes used to quantitatively determine HDL cholesterol may be used as they are for quantitative determination of LDL cholesterol or cholesterol other than HDL or total cholesterol.

Alternatively, for the quantitative determination of LDL cholesterol or cholesterol other than HDL or total cholesterol, CH enzymes having the same or different specificities may be newly added to the system.

Preferably, chemically modified CH enzymes are used for the quantitative determination of HDL cholesterol and for the determination of cholesterol in lipoproteins other than LDL or HDL or total cholesterol, CH enzymes without any chemical modification are used.

In the reaction of cholesterol according to the present invention, a surfactant or cholic acid which is conventionally used to activate CH enzymes may also be employed as far as they do not affect the reaction specificity. Further, various salts for solubilizing proteins such as globulin may also be used.

As the surfactant for activating the CH enzymes, anionic surfactants are used, e.g., at a concentration of 0 to 1%. Examples of the cholic acid are cholic acid, deoxycholic acid, taurocholic acid and chenodeoxycholic acid. The cholic acid is used at a concentration of 0 to 5%. Examples of the anionic surfactant include an alkyl sulfonate such as 1-pentasulfonate, 1-hexasulfonate, 1-heptasulfonate and 1-octasulfonate. These surfactants are used at a concentration of 0 to 5%.

Examples of the salts include sodium chloride, sodium sulfate, potassium chloride, potassium sulfate, magnesium chloride, magnesium sulfate, magnesium acetate, lithium chloride, lithium sulfate, ammonium chloride, ammonium sulfate, magnesium nitrate and calcium nitrate. These salts are used at a concentration of 0 to 100 mM.

When the reaction of cholesterol is carried out with cholesterol esterase and cholesterol oxidase, hydrogen peroxide is formed. The formed hydrogen peroxide can be quantitatively determined, using e.g. 4-aminoantipyrine and a phenol, 4-aminoantipyrine and Trinder's reagent, or a highly sensitive chromogen in the presence of peroxidase.

Examples of phenols are phenol, 4-chlorophenol, m-cresol and 3-hydroxy-2,4,6-triiodobenzoic acid (HTIB).

Examples of the Trinder's reagents (General Catalog of Dojin Kagaku Kenkyusho, 19th ed., 1994) are anilines such as N-sulfopropylaniline, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS), N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethylaniline (MAOS), N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (DAOS), N-ethyl-N-sulfopropyl-m-toluidine (TOPS), N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (HDAOS), N,N-dimethyl-m-toluidine, N,N-disulfopropyl-3,5-dimethoxyaniline, N-ethyl-N-sulfopropyl-m-anisidine, N-ethyl-N-sulfopropylaniline, N-ethyl-N-sulfopropyl-3,5-dimethoxyaniline, N-sulfopropyl-3,5-dimethoxyaniline, N-ethyl-N-sulfopropyl-3,5-dimethylaniline, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-anisidine, N-ethyl-N-(2-hydroxy-3-sulfopropyl)aniline and N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, N-ethyl-N-(3-methylphenyl)-N'-succinylethylenediamine (EMSE), and N-ethyl-N-(3-methylphenyl)-N'-acetylethylenediamine.

As the highly sensitive chromogen, there are 10-(N-methylcarbamoyl)-3,7-bis(dimethylamino)phenothiadine (MCDP) disclosed in Japanese Published Examined Patent Application No. 33479/85, bis[3-bis(4-chlorophenyl)methyl-4-dimethylaminophenyl]amine (BCMA) disclosed in Japanese Published Examined Patent Application No. 27839/92, the compounds disclosed in Japanese Published Unexamined Patent Application No. 296/87, etc.

The chromogen is preferably used in a concentration of 0.01 to 10 mg/ml.

When the reaction of cholesterol is carried out with cholesterol esterase and cholesterol dehydrogenase in the presence of an oxidized coenzyme, NAD(P), as a substrate, a reduced coenzyme, NAD(P)H, is formed. The formed NAD(P)H can be quantitatively determined by measuring the absorbance of a reaction mixture at 300 to 500 nm, preferably 330 to 400 nm, particularly preferably about 340 nm. The determination of NAD(P)H may otherwise be made by forming a formazan pigment

through addition of diaphorase and a tetrazolium salt and then measuring the formazan pigment by colorimetry.

The reaction for the quantitative determination of LDL cholesterol is carried out at 10 to 50℃, preferably 30 to 40℃, usually at 37℃, for 1 to 30 minutes, preferably 2 to 10 minutes.

In the fractional determination, the reaction for quantitatively determining HDL cholesterol (hereinafter referred to as the first reaction) are carried out at 10 to 50℃, preferably 30 to 40℃, usually at 37℃, for 1 to 30 minutes, preferably 2 to 10 minutes; the reactions for quantitatively determining LDL cholesterol or total cholesterol (hereinafter referred to as the second reaction) are carried out at 10 to 50℃, preferably 30 to 40℃, usually at 37℃, for 1 to 30 minutes, preferably 2 to 10 minutes. The start of the second reaction may be at any stage, e.g., after the first reaction is substantially completed or during the first reaction, so long as the quantitative determination of HDL is completed. The second reaction is initiated by adding the reagent A enabling the CH enzymes to act specifically on LDL cholesterol or the reagent C enabling the CH enzymes to act on cholesterol in all lipoproteins and, if necessary, CH enzymes. The hydrogen peroxide or reduced coenzyme [NAD(P)H] thus formed by the second reaction is quantitatively determined using the same reagents as used in the first reaction as they are, or, if necessary and desired, reagents may be newly added to the system.

In the present invention where HDL cholesterol and LDL cholesterol are fractionally determined by first performing the reaction of HDL cholesterol followed by the reaction of LDL cholesterol, the reaction of LDL cholesterol is initiated by adding the reagent A as described above. In this case, when the first reaction of HDL cholesterol is carried out by adding the nonionic surfactant that does not dissolve the aggregated lipoproteins other than HDL, i.e., by adding either the polyoxyethylene derivative or the polyoxyethylene-polyoxypropylene copolymer, the second reaction of LDL cholesterol may also be initiated by adding such a surfactant

as forming the reagent A in combination with the surfactant used in the reaction of HDL cholesterol.

The concentration of cholesterol in each lipoprotein is calculated by the following equation based on a difference in absorbance (ΔOD) before and after each reaction using a test sample and a difference in absorbance (ΔODstd) using a sample with a known concentration of cholesterol in various lipoproteins.

The concentration of LDL cholesterol can be determined by the following equation:

$\Delta OD \div \Delta ODstd \times$ (known concentration of LDL cholesterol)

The concentration of HDL cholesterol can be determined by, e.g., the following equation:

$\Delta OD \div \Delta ODstd \times$ (known concentration of HDL cholesterol)

In the fractional determination, when the compounds formed in the first and second reactions are the same and they are detected by the same method, the concentration of total cholesterol can be calculated according to the following equation, using the difference in absorbance before the first reaction and after the second reaction:

$\Delta OD \div \Delta ODstd \times$ (known concentration of total cholesterol)

The reagent of the present invention for quantitatively determining LDL cholesterol comprises CH enzymes and a reagent comprising the polyoxyethylene derivative and the polyoxyethylene-polyoxypropylene copolymer. The above reagent for quantitatively determining LDL cholesterol may further contain, if necessary, the aforesaid buffers, reagents for aggregating lipoproteins other than HDL, surfactants used for quantitatively determining cholesterol, cholic acids, various salts, enzymes such as peroxidase, chromogens such as 4-aminoantipyrine and Trinder's reagents or oxidized coenzymes such as NAD(P).

The reagent kit of the present invention for the fractional determination of HDL cholesterol and LDL cholesterol comprises a first reagent and a second reagent. For example,

the first reagent comprises a reagent containing an aggregating agent for lipoproteins other than HDL and CH enzymes and the second reagent comprises a reagent containing the polyoxyethylene derivative and the polyoxyethylene-polyoxypropylene copolymer.

The reagent kit of the present invention for the fractional determination of HDL cholesterol and total cholesterol comprises a first reagent and a second reagent. For example, the first reagent comprises a reagent containing an aggregating agent for lipoproteins other than HDL and CH enzymes and the second reagent comprises a reagent containing a nonionic surfactant that dissolves all lipoproteins (HDL, LDL, VLDL and CM).

The first and second reagents of the reagent kit in accordance with the present invention may further contain, if necessary and desired, the aforesaid buffers, surfactants used for the quantitative determination of cholesterol, cholic acids, various salts, enzymes such as peroxidase, chromogens such as 4-aminoantipyrine and Trinder's reagents, oxidized coenzymes such as NAD(P).

In the second reagent, the source of CH enzymes may be the same as or different from the first reagent. It is preferred that the chemically modified enzyme described above is used as the CH enzyme for the first reagent and a CH enzyme not chemically modified is used as the CH enzyme for the second reagent.

Brief Description of the Drawings

FIG. 1 is a graph showing the correlation between the concentration of HDL cholesterol obtained by the method of the present invention (designated by DB HDL-C in the figure) and the concentration of HDL cholesterol obtained by the comparative method (L HDL-C method, designated by S HDL-C in the figure).

FIG. 2 is a graph showing the correlation between the concentration of LDL cholesterol obtained by the method of the present invention (designated by DB LDL-C in the figure) and

the concentration of LDL cholesterol obtained by the comparative method (L LDL-C method, designated by S LDL-C in the figure).

FIG. 3 is a graph showing the correlation between the concentration of total cholesterol obtained by the method of the present invention (designated by DB-TC in the figure) and the concentration of total cholesterol obtained by the comparative method (Determiner L TC II method, designated by L TC II in the figure).

FIG. 4 is a graph showing the correlation between the concentration of HDL cholesterol obtained by the method of the present invention (designated by DB HDL-C in the figure) and the concentration of HDL cholesterol obtained by the comparative method (L HDL-C method, designated by S HDL-C in the figure).

FIG. 5 is a graph showing the correlation between the concentration of LDL cholesterol obtained by the method of the invention (designated by Method of the invention in the figure) and the concentration of LDL cholesterol obtained by the comparative method (designated by Comparative method in the figure) which is calculated in accordance with the Friedewald formula of conversion.

Best Modes for Carrying Out the Invention

Example 1 Determination of HDL cholesterol and LDL cholesterol

First reagent (pH = 7)

MES (Nacalai Tesque, Inc.)	20 mM
Dextran sulfonic acid (Tokyo Kasei)	0.23 mg/ml
Magnesium sulfate (Kanto Chemical Co., Ltd.)	1.5 mg/ml
HDAOS (Dojin Kagaku)	0.23 mg/ml
4-Aminoantipyrine (Saikyo Kasei)	0.13 mg/ml
Polyethylene glycol-modified cholesterol esterase(*1)	0.25 U/ml
Polyethylene glycol-modified cholesterol oxidase(*2)	1.65 U/ml

Peroxidase(Toyobo Co., Ltd.)	12.5 U/ml

(*1): Prepared by dissolving 50 g of cholesterol esterase (Amano Pharmaceutical Co. Ltd.) in 1 L of 0.1 M HEPES buffer (pH8.5) and adding 330 g of Sun Bright VFM4101 to the solution at 25°C, followed by stirring for 2 hours.

(*2): Prepared by dissolving 50 g of cholesterol oxidase (Kyowa Hakko Kogyo Co., Ltd.) in 1 L of 0.1 M HEPES buffer (pH8.0) and adding 10 g of Sun Bright VFM4101 to the solution at 15°C, followed by stirring for 2 hours.

Second reagent (pH = 7)

MES (Nacalai Tesque, Inc.)	20 mM
Cholesterol esterase (lipoprotein lipase, Toyobo Co., Ltd.)	3 U/ml
Cholesterol oxidase(Kyowa Hakko Kogyo Co., Ltd.)	2 U/ml
Pulronic L-121 (Asahi Denka Kogyo K.K.)	0.7%
Emulgen L-40 (Kao Corporation)	0.5 %
Calcium chloride (Wako Pure Chemical Industries, Ltd.)	0.1 mg/ml

Serum samples from 30 healthy subjects were prepared and HDL cholesterol and LDL cholesterol in the samples were determined by the following procedures.

Reagent 1, 2.25 ml, was mixed with 30 μl of the sample. After the mixture was stirred, absorbance E1 was immediately measured at 585 nm. The mixture was then incubated at 37℃ for 5 minutes, and absorbance E2 at the same wavelength was measured. To the reaction solution was added 0.75 ml of Reagent 2. After the mixture was stirred, absorbance E3 was immediately measured at 585 nm and after the mixture was incubated at 37℃ for 5 minutes, absorbance E4 was measured at the same wavelength. Sera having known concentration of cholesterol were treated by substantially the same procedure to measure absorbances E1std, E2std, E3std and E4std, respectively.

The concentration of HDL cholesterol was determined by the following equation, using the absorbance data.

(E2-E1)÷(E2std-E1std) x (known concentration of HDL cholesterol)

The concentration of LDL cholesterol was likewise determined by the following equation, using the absorbance data.

(E4-E3)÷(E4std-E3std) x (known concentration of LDL cholesterol)

For comparison, the concentration of HDL cholesterol and LD cholesterol in each serum sample was determined using Determiner L HDL-C and Determiner L LDL-C (both manufactured by Kyowa Medex Co., Ltd.), which are commercial kits for independent determination of the cholesterol, respectively. The coefficient of correlation between the results obtained with these commercial kits and the results according to the method of the present invention was calculated. The coefficient of correlation showed 0.997 for the HDL cholesterol and 0.988 for the LDL cholesterol.

Fig. 1 shows a correlation between the concentration (mg/dL) of HDL cholesterol according to the method of this invention (designated as DB HDL-C in Fig. 1) and the concentration (mg/dL) of HDL cholesterol obtained by the comparative method (L HDL-C method, designated as S HDL-C in Fig. 1). Fig. 2 shows a correlation between the concentration (mg/dL) of LDL cholesterol according to the method of this invention (designated as DB LDL-C in Fig. 2) and the concentration (mg/dL) of LDL cholesterol obtained by the comparative method (L LDL-C method, designated as S LDL-C in Fig. 2).

Example 2 Determination of HDL cholesterol and LDL cholesterol

First reagent (pH = 7)

MES (Nacalai Tesque, Ltd.)	20 mM
Phosphotungstic acid (Wako Pure Chemical Industries, Ltd.)	7.5 mg/ml

Magnesium sulfate (Wako Pure Chemical Industries, Ltd.)	1.5 mg/ml
TOOS (Dojin Kagaku)	0.5 mg/ml
Emulgen B66 (Kao Corporation)	10 mg/ml
4-Aminoantipyrine (Saikyo Kagaku)	0.5 mg/ml
Cholesterol esterase (LPBP, Asahi Chemical Industry Co., Ltd.)	4 U/ml
Cholesterol oxidase (rCO, Oriental Yeast Co., Ltd.)	2 U/ml
Peroxidase (Toyobo Co., Ltd.)	10 U/ml

Second reagent (pH = 7)

MES (Nacalai Tesque, Inc.)	50 mM
Cholesterol esterase(lipoprotein lipase, Toyobo Co., Ltd.)	3 U/ml
Cholesterol oxidase(Kyowa Hakko Kogyo Co., Ltd.)	2 U/ml
Pulronic L-121 (Asahi Denka Kogyo K.K.)	0.7%
Emulgen L-40 (Kao Corporation)	0.5%
Calcium chloride (Wako Pure Chemical Industries, Ltd.)	0.1 mg/ml

To determine HDL cholesterol and LDL cholesterol, substantially the same procedure as in Example 1 was repeated using the same samples as in Example 1 except that the wavelength measured was changed to 555 nm. The coefficient of correlation between the results obtained with the commercial kits of Determiner L HDL-C and Determiner L HDL-C and the results obtained according to the method of the present invention was calculated. The coefficient of correlation showed 0.929 for the HDL cholesterol and 0.911 for the LDL cholesterol.

Example 3 Determination of HDL cholesterol and total cholesterol

First reagent (pH = 7)

MES (Nacalai Tesque, Inc.)	20 mM
Dextran sulfonic acid (Tokyo Kasei)	0.23 mg/ml

Magnesium sulfate (Kanto Chemical Co., Ltd.)	1.5 mg/ml
HDAOS (Dojin Kagaku)	0.23 mg/ml
4-Aminoantipyrine (Saikyo Kasei)	0.13 mg/ml
Polyethylene glycol-modified cholesterol esterase (*1)	0.25 U/ml
Polyethylene glycol-modified cholesterol oxidase (*1)	1.65 U/ml
Peroxidase	12.5 U/ml

(*1): Prepared by the same procedure as *1 in Example 1

(*2): Prepared by the same procedure as *2 in Example 1

Second reagent (pH = 6.75)

MES (Nacalai Tesque, Inc.)	30 mM
Triton X-100 (Sigma)	1 g/L
Cholesterol esterase (Toyobo Co., Ltd.)	2.4 U/ml
Cholesterol oxidase (Amano Pharmaceutical Co., Ltd.)	6.25 U/ml

Serum samples from 30 healthy subjects used in Example 1 were prepared and HDL cholesterol and LDL cholesterol of the samples were determined by the following procedures.

Reagent 1, 2.25 ml, was mixed with 30 μl of the sample. After the mixture was stirred, absorbance E1 was immediately measured at 585 nm. The mixture was then incubated at 37℃ for 5 minutes, and absorbance E2 was measured at the same wavelength. To the reaction solution was further added 0.75 ml of Reagent 2. After the mixture was stirred, absorbance E3 was immediately measured at 585 nm and after the mixture was incubated at 37℃ for 5 minutes, absorbance E4 was measured at the same wavelength. Separately, sera having known concentration of cholesterol were treated by substantially the same procedure to measure absorbances E1std, E2std, E3std and E4std, respectively.

The concentration of HDL cholesterol was determined by the following equation, using the absorbance data.

$$(E2-E1) \div (E2std-E1std) \times (\text{known concentration of HDL cholesterol})$$

The concentration of the total cholesterol was also determined by the following equation, using the absorbance data.

(E4-E1)÷(E4std-E1std) x (known concentration of the total cholesterol)

For comparison, the concentration of HDL cholesterol and the total cholesterol in each serum sample was determined using Determiner L HDL-C and Determiner L TC II (both manufactured by Kyowa Medex Co., Ltd.), which are commercial kits for independent determination of the cholesterol, respectively. The coefficient of correlation between the results obtained with the commercial kits and the results according to the method of the present invention was calculated. The coefficient of correlation showed 0.992 for the HDL cholesterol and 0.999 for the total cholesterol.

Fig. 3 shows a correlation between the concentration (mg/dL) of the total cholesterol according to the method of this invention (designated as DB-TC in Fig. 3) and the concentration (mg/dL) of the total cholesterol obtained by the comparative method (Determiner L TC II method, designated as L TC II in Fig. 3).

Fig. 4 shows a correlation between the concentration (mg/dL) of HDL cholesterol according to the method of this invention (designated as DB HDL-C in Fig. 4) and the concentration (mg/dL) of HDL cholesterol obtained by the comparative method (L HDL-C method, designated as S HDL-C in Fig. 4).

Example 4

First reagent (pH 7.25)

PIPES (Nacalai Tesque, Inc.)	50 mM
HDAOS (Dojin Kagaku)	0.3 mg/mL

Second reagent (pH 7.25)

PIPES (Nacalai Tesque, Inc.)	50 mM
Cholesterol esterase (lipoprotein lipase, Toyobo Co., Ltd.)	5 U/mL

Cholesterol oxidase (Kyowa Hakko Kogyo Co., Ltd.)	1 U/mL
Peroxidase (Toyobo Co., Ltd.)	20 U/mL
4-Aminoantipyrine (Saikyo Kasei)	0.51 mg/mL
Calcium chloride (Wako Pure Chemical Industries, Ltd.)	0.1 mg/mL
Surfactant (kind and concentration given in Table 1)	

As samples, HDL, LDL, VLDL and CM separated from human blood serum by the ultracentrifugation method were used. The respective lipoprotein fractions were provided by Fukushi Iryo Gijutsu Shinkoukai (Welfare Medical Technology Promotion Organization). These fractions were prepared in accordance with Adv. Lipid Res., 6 (1968) [Practical methods for plasma lipoprotein analysis by Hatch, F. & Lees, R.]. The concentration of cholesterol in each lipoprotein used in this test was determined using Determiner L TC II (Kyowa Medex Co., Ltd.). The concentration was found to be 73 mg/dL for HDL, 264 mg/dL for LDL, 84 mg/dL for VLDL and 17 mg/dL for CM.

After 4 μL of each sample was mixed with 300 μL of the first reagent, the mixture was maintained at 37℃ for 5 minutes. At this stage, an absorbance of the mixture was measured. Thereafter, 100 μL of the second reagent was added to the mixture and reacted. After 5 minutes, an absorbance of the reaction mixture was measured. The absorbance was measured at a principal wavelength of 600 nm and a secondary wavelength of 700 nm, using Hitachi 7070 autoanalyzer.

Differences in absorbance obtained before and after the reactions using LDL fraction, HDL fraction, VLDL fraction and CM fraction are shown by A_{LDL}, A_{HDL}, A_{VLDL} and A_{CM}, respectively.

The results are shown in Table 1 in terms of A_{HDL}/A_{LDL}, A_{VLDL}/A_{LDL} and A_{CM}/A_{LDL}, respectively. The results mean that as the ratio becomes smaller, the conditions for quantitative determination are more specific to LDL.

Table 1

Surfactant	Concentration (%)	A_{HDL}/A_{LDL}	A_{VLDL}/A_{LDL}	A_{CM}/A_{LDL}
Pluronic L-121 Emulgen L40	0.2 0.16	7.3	6.6	4.6
Pluronic L-121 Nonion HS-210	0.2 0.1	9.6	13.5	3.2
Pluronic L-121 Emulgen 911	0.2 0.1	10.2	7.7	1.2
Pluronic L-122 Emulgen L40	0.2 0.16	8.1	8.2	3.4
Pluronic L-121 (comparative example 1)	0.2	34.7	47.9	16.8
Emulgen L-40 (comparative example 2)	0.16	27.8	39.7	9.7
Nonion HS-210 (comparative example 3)	0.1	35.5	35.5	6.1
Nonion HS-215 (comparative example 4)	0.16	76.8	33.6	4.7
Nonion NS-208.5 (comparative example 5)	0.24	44.5	32.4	51.2
Nonion NS-208 (comparative example 6)	0.08	30.2	47.3	28.3
Emulgen 911 (comparative example 7)	0.1	22.6	15.9	3.0
Emulgen 810 (comparative example 8)	0.2	24.7	36.8	5.8
Pluronic L-122 (comparative example 9)	0.2	38.1	64.1	19.0

As shown in Table 1, the results reveal that by using the surfactants in combination, the reaction of cholesterol is more specific to LDL cholesterol than the case of using the surfactant alone.

Example 5

First reagent (pH 6.75)

MOPS (Nacalai Tesque, Inc.)	50 mM
HDAOS (Dojin Kagaku)	0.3 mg/mL

Second reagent (pH 6.75)

MOPS (Nacalai Tesque, Inc.)	50 mM
Cholesterol esterase (lipoprotein lipase, Toyobo Co., Ltd.)	1 U/mL
Cholesterol oxidase (Kyowa Hakko Kogyo Co., Ltd.)	3 U/mL
Peroxidase (Toyobo Co., Ltd.)	20 U/mL
4-Aminoantipyrine (Saikyo Kasei)	0.51 mg/mL
Calcium chloride (Wako Pure Chemical Industries, Ltd.)	0.1 mg/mL
Surfactant (kind and concentration given in Table 2)	

The test was carried out in a manner similar to Example 4 except for using the surfactants shown in Table 2. A_{LDL}, A_{HDL}, A_{VLDL} and A_{CM} were thus determined, respectively, based on which the ratios of A_{HDL}/A_{LDL}, A_{VLDL}/A_{LDL} and A_{CM}/A_{LDL} were calculated. The concentration of cholesterol in each lipoprotein used in this test was determined using Determiner L TC II (Kyowa Medex Co., Ltd.) and was found to be 81 mg/dL for HDL, 263 mg/dL for LDL, 72 mg/dL for VLDL and 14 mg/dL for CM.

The results are shown in Table 2.

Table 2

Surfactant	Concentration (%)	A_{HDL}/A_{LDL}	A_{VLDL}/A_{LDL}	A_{CM}/A_{LDL}
Pluronic L-101 Emulgen L-40	0.2 0.16	8.7	7.3	2.6
Pluronic P-103 Emulgen L-40	0.2 0.16	13.0	3.9	1.7
Pluronic F-108 Emulgen L-40	0.2 0.16	15.0	4.5	1.4
Emulgen L-40 (comparative example 10)	0.16	26.5	24.6	4.7
Pluronic L-101 (comparative example 11)	0.2	19.0	14.3	5.6
Pluronic P-103 (comparative example 12)	0.2	24.8	3.5	1.1
Pluronic F-108 (comparative example 13)	0.2	28.8	17.8	1.6

As shown in Table 2, the results reveal that by using the surfactants in combination, the reaction of cholesterol is more specific to LDL cholesterol than the case of using the surfactant alone.

Example 6

First reagent (pH 6.75)

MOPS (Nacalai Tesque, Inc.)	20 mM
HDAOS (Dojin Kagaku)	0.3 mg/mL

Second reagent (pH 6.75)

MOPS (Nacalai Tesque, Inc.)	20 mM
Cholesterol esterase (lipoprotein lipase, Toyobo Co., Ltd.)	2 U/mL
Cholesterol oxidase (Kyowa Hakko Kogyo Co., Ltd.)	3 U/mL
Peroxidase (Toyobo Co., Ltd.)	20 U/mL
4-Aminoantipyrine (Saikyo Kasei)	0.51 mg/mL

Calcium chloride (Wako Pure Chemical Industries, Ltd.) 0.1 mg/mL
Surfactant (kind and concentration given in Table 3)

The test was carried out in a manner similar to Example 4 except for using the surfactants shown in Table 3. A_{LDL}, A_{HDL}, A_{VLDL} and A_{CM} were determined, respectively, based on which the ratios of A_{HDL}/A_{LDL}, A_{VLDL}/A_{LDL} and A_{CM}/A_{LDL} were calculated. The concentration of cholesterol in each lipoprotein used in this test was determined with Determiner L TC II (manufactured by Kyowa Medex Co., Ltd.) and was found to be 85 mg/dL for HDL, 252 mg/dL for LDL, 75 mg/dL for VLDL and 19 mg/dL for CM.

The results are shown in Table 3.

Table 3

Surfactant	Concentration (%)	A_{HDL}/A_{LDL}	A_{VLDL}/A_{LDL}	A_{CM}/A_{LDL}
Pluronic L-121 Emulgen L-40	0.7 0.5	4.0	5.0	3.4

As shown in Table 3, LDL cholesterol can be more specifically determined by using the combination of surfactants.

Example 7

First reagent (pH 7.0)
MOPS (Nacalai Tesque, Inc.) 10 mM
HDAOS (Dojin Kagaku) 0.3 mg/mL
Second reagent (pH 7.0)
MOPS (Nacalai Tesque, Inc.) 50 mM
Cholesterol esterase (lipoprotein lipase, Toyobo Co., Ltd.) 1 U/mL
Cholesterol oxidase (Kyowa Hakko Kogyo Co., Ltd.) 3 U/mL
Peroxidase (Toyobo Co., Ltd.) 20 U/mL
4-Aminoantipyrine (Saikyo Kasei) 0.51 mg/mL

Calcium chloride (Wako Pure Chemical Industries, Ltd.) 0.1 mg/mL
Surfactant (kind and concentration given in Table 4)

The test was carried out in a manner similar to Example 4 except for using the surfactants shown in Table 4. A_{LDL}, A_{HDL}, A_{VLDL} and A_{CM} were determined, respectively, based on which the ratios of A_{HDL}/A_{LDL}, A_{VLDL}/A_{LDL} and A_{CM}/A_{LDL} were calculated. The concentration of cholesterol in each lipoprotein used in this test was determined using Determiner L TC II (Kyowa Medex Co., Ltd.) and was found to be 79 mg/dL for HDL, 273 mg/dL for LDL, 76 mg/dL for VLDL and 16 mg/dL for CM.

The results are shown in Table 4.

Table 4

Surfactant	Concentration (%)	A_{HDL}/A_{LDL}	A_{VLDL}/A_{LDL}	A_{CM}/A_{LDL}
Pluronic L-121 Emulgen L-40	0.4 0.32	2.5	5.8	1.3

As shown in Table 4, LDL cholesterol can be determined more specifically by using the combination of the surfactants.

Example 8

First reagent (pH 7.0)
MOPS (Nacalai Tesque, Inc.) 10 mM
HDAOS (Dojin Kagaku) 0.3 mg/mL
Magnesium chloride hexahydrate (Kanto Chemical Co., Ltd.) 7 mg/dL
Sodium dextran sulfate (Tokyo Kasei) 0.7 mg/dL
Second reagent (pH 6.75)
MOPS (Nacalai Tesque, Inc.) 50 mM
Cholesterol esterase (lipoprotein lipase, Toyobo Co., Ltd.) 1 U/mL
Cholesterol oxidase (Kyowa Hakko Kogyo Co., Ltd.) 3 U/mL

Peroxidase (Toyobo Co., Ltd.)	20 U/mL
4-Aminoantipyrine (Saikyo Kasei)	0.51 mg/mL
Calcium chloride (Wako Pure Chemical Industries, Ltd.)	0.1 mg/mL
Surfactant (kind and concentration given in Table 5)	

The test was carried out in a manner similar to Example 4 except that the surfactants shown in Table 4 were used and the absorbance was measured immediately after the addition of the second reagent and 5 minutes after the addition of the second reagent to obtain the differences in the absorbance as A_{LDL}, A_{HDL}, A_{VLDL} and A_{CM}, respectively. Based on the differences, the ratios of A_{HDL}/A_{LDL}, A_{VLDL}/A_{LDL} and A_{CM}/A_{LDL} were calculated. The concentration of cholesterol in each lipoprotein used in this test was determined using Determiner L TC II (Kyowa Medex Co., Ltd.) and was found to be 79 mg/dL for HDL, 273 mg/dL for LDL, 76 mg/dL for VLDL and 16 mg/dL for CM.

The results are shown in Table 5.

Table 5

Surfactant	Concentration (%)	A_{HDL}/A_{LDL}	A_{VLDL}/A_{LDL}	A_{CM}/A_{LDL}
Pluronic L-121 Emulgen L-40	0.4 0.32	2.6	4.6	1.3

As shown in Table 5, LDL cholesterol can be determined more specifically by using the combination of the surfactants.

Example 9

First reagent (pH 7.0)

MOPS (Nacalai Tesque, Inc.)	10 mM
HDAOS (Dojin Kagaku)	0.3 mg/mL
Magnesium chloride hexahydrate (Kanto Chemical Co., Ltd.)	7 mg/dL
Sodium dextran sulfate (Tokyo Kasei)	0.7 mg/dL

Second reagent (pH 7.0)

MOPS (Nacalai Tesque, Inc.) 50 mM
Cholesterol esterase (lipoprotein lipase, Toyobo Co., Ltd.) 1 U/mL
Cholesterol oxidase (Kyowa Hakko Kogyo Co., Ltd.) 0.6 U/mL
Peroxidase (Toyobo Co., Ltd.) 20 U/mL
4-Aminoantipyrine (Saikyo Kasei) 0.51 mg/mL
Calcium chloride (Wako Pure Chemical Industries Ltd.) 0.1 mg/mL
Surfactant (kind and concentration given in Table 6)

The test was carried out in a manner similar to Example 8 except for using the surfactants shown in Table 6. A_{LDL}, A_{HDL}, A_{VLDL} and A_{CM} were determined, respectively, based on which the ratios of A_{HDL}/A_{LDL}, A_{VLDL}/A_{LDL} and A_{CM}/A_{LDL} were calculated. The concentration of cholesterol in each lipoprotein used in this test was determined using Determiner L TC II (Kyowa Medex Co., Ltd.) and was found to be 82 mg/dL for HDL, 270 mg/dL for LDL, 73 mg/dL for VLDL and 14 mg/dL for CM.

The results are shown in Table 6.

Table 6

Surfactant	Concentration (%)	A_{HDL}/A_{LDL}	A_{VLDL}/A_{LDL}	A_{CM}/A_{LDL}
Pluronic L-121 Emulgen L-40	0.375 0.5	2.5	4.3	1.4
Pluronic L-121 Emulgen L-40	0.7125 0.57	2.5	2.2	1.8

As shown in Table 6, LDL cholesterol can be determined more specifically by using the combination of the surfactants.

Example 10

First reagent (pH 7.25)
PIPES (Nacalai Tesque, Inc.) 50 mM
HDAOS (Dojin Kagaku) 0.3 mg/mL

Second reagent (pH 7.25)

PIPES (Nacalai Tesque, Inc.)	50 mM
Cholesterol esterase (lipoprotein lipase, Toyobo Co., Ltd.)	2 U/mL
Cholesterol oxidase (Kyowa Hakko Kogyo Co., Ltd.)	3 U/mL
Peroxidase (Toyobo Co., Ltd.)	20 U/mL
4-Aminoantipyrine (Saikyo Kasei)	0.51 mg/mL
Calcium chloride (Wako Pure Chemical Industries, Ltd.)	0.1 mg/mL
Emulgen L-40 (Kao Corporation)	0.16%
Pluronic L-121 (Asahi Denka Kogyo K. K.)	0.2%

As human serum samples, 88 samples were collected from the patients and provided for the quantitative determination of LDL cholesterol in the samples according to the following procedures.

After 4 μL of each sample was mixed with 300 μL of the first reagent, the mixture was kept at 37℃ for 5 minutes. At this stage, an absorbance of the mixture was measured. Thereafter, 100 μL of the second reagent was added to the mixture and reacted. After 5 minutes, an absorbance of the reaction mixture was measured. Separately, sera with known concentrations of LDL cholesterol were treated, respectively, in the same manner. By measuring an absorbance, the concentration of cholesterol in each sample was quantitatively determined. The absorbance was measured at a principal wavelength of 600 nm and a secondary wavelength of 700 nm, using Hitachi 7070 autoanalyzer.

On the other hand, total cholesterol, HDL cholesterol and neutral fat were measured using Determiner L TC (Kyowa Medex Co., Ltd.), Determiner L HDL-C (Kyowa Medex Co., Ltd.) and Determiner L TG (Kyowa Medex Co., Ltd.), respectively, which are all commercially available kits. Then the concentration of LDL cholesterol was determined in accordance with the following Friedewald formula. A correlation coefficient

between the concentration of LDL cholesterol obtained by the method of the present invention and the concentration of LDL cholesterol calculated according to the Friedewald formula was found to be 0.9767.

Friedewald formula:

(concentration of LDL cholesterol) = (concentration of total cholesterol) - (concentration of HDL cholesterol) - (concentration of neutral fat)

FIG. 5 shows the correlation between the concentration of LDL cholesterol obtained by the method of the invention (designated by Method of the invention in the figure) and the concentration of LDL cholesterol obtained by the comparative method (designated by Comparative method in the figure).

Industrial Applicability

The present invention provides the method for the quantitative determination of LDL cholesterol and the reagent kit for use in the method. The present invention also provides the method for continuous fractional determination of HDL cholesterol and LDL cholesterol or total cholesterol in the same sample in the same system, as well as a reagent kit for use therein.

Claims

(1) A method for quantitatively determining LDL cholesterol in a biological sample, which comprises performing the reaction of cholesterol in the presence of:

a) a biological sample,

b) cholesterol esterase and cholesterol oxidase or cholesterol dehydrogenase (hereinafter collectively referred to as CH enzymes), and

c) a reagent enabling the CH enzymes of b) to act only on LDL cholesterol, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the reaction to quantitatively determine the concentration of LDL cholesterol.

(2) The method according to claim (1), wherein the reagent enabling CH enzymes to act only on LDL cholesterol is a reagent containing at least a polyoxyethylene derivative and a polyoxyethylene-polyoxypropylene copolymer.

(3) The method according to claim (2), wherein the polyoxyethylene derivative is a polyoxyethylene alkyl ether or a polyoxyethylene alkylaryl ether.

(4) The method according to claim (2) or (3), wherein the polyoxyethylene-polyoxypropylene copolymer is a surfactant represented by general formula (I):

$$HO-(C_2H_4O)_a-(C_3H_6O)_b-(C_2H_4O)_c-H \qquad (I)$$

(wherein a, b and c, which may be the same or different, each represents an integer of 1 to 200).

(5) A method for the continuous fractional determination of HDL cholesterol and LDL cholesterol in a biological sample, which comprises

subjecting cholesterol to the first reaction in the presence of:

a) a biological sample,

b) CH enzymes, and

c) a reagent enabling the CH enzymes of b) to act only on HDL cholesterol, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the first reaction to quantitatively determine the concentration of HDL cholesterol,

then adding

d) a reagent enabling the CH enzymes of b) to act only on LDL cholesterol,

subjecting cholesterol to the second reaction, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the second reaction to quantitatively determine the concentration of LDL cholesterol.

(6) A method for the continuous fractional determination of HDL cholesterol and LDL cholesterol in a biological sample, which comprises

subjecting cholesterol to the first reaction in the presence of:

a) a biological sample,

b) CH enzymes, and

c) a reagent enabling the CH enzymes of b) to act only on HDL cholesterol, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the first reaction to quantitatively determine the concentration of HDL cholesterol,

then adding

d) CH enzymes, and

e) a reagent enabling the CH enzymes of d) to act only on LDL cholesterol,

subjecting cholesterol to the second reaction, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the second reaction to quantitatively determine the concentration of LDL cholesterol.

(7) The method according to claim (5) or (6), wherein the reagent enabling CH enzymes to act only on LDL cholesterol is a reagent containing at least a polyoxyethylene derivative and a polyoxyethylene-polyoxypropylene copolymer.

(8) The method according to claim (7), wherein the polyoxyethylene derivative is a polyoxyethylene alkyl ether or a polyoxyethylene alkylaryl ether.

(9) The method according to claim (7) or (8), wherein the polyoxyethylene-polyoxypropylene copolymer is a surfactant represented by general formula (I):

$$HO-(C_2H_4O)_a-(C_3H_6O)_b-(C_2H_4O)_c-H \qquad (I)$$

(wherein a, b and c, which may be the same or different, each represents an integer of 1 to 200).

(10) A method for the continuous fractional determination of HDL cholesterol and total cholesterol in a biological sample, which comprises

subjecting cholesterol to the first reaction in the presence of:

a) a biological sample,

b) CH enzymes, and

c) a reagent enabling CH enzymes of b) to act only on HDL cholesterol, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the first reaction to quantitatively determine the concentration of HDL cholesterol,

then adding

d) a reagent enabling the CH enzymes of b) to act on cholesterol in all lipoproteins,

subjecting cholesterol to the second reaction, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the second reaction to quantitatively determine the concentration of total cholesterol.

(11) A method for the continuous fractional determination of HDL cholesterol and total cholesterol in a biological sample, which comprises

subjecting cholesterol to the first reaction in the presence of:

a) a biological sample,

b) CH enzymes, and

c) a reagent enabling the CH enzymes of b) to act only on HDL cholesterol, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the first reaction to quantitatively determine the concentration of HDL cholesterol,

then adding

d) CH enzymes, and

e) a reagent enabling the CH enzymes of d) to act on cholesterol in all lipoproteins,

subjecting cholesterol to the second reaction, and

measuring the amount of the hydrogen peroxide or reduced coenzyme formed by the second reaction to quantitatively determine the total cholesterol.

(12) The method according to any one of claims (5) through (11), wherein the reagent enabling CH enzymes to act only on cholesterol in HDL is a reagent for aggregating lipoproteins other than HDL.

(13) The method according to claim (12), wherein the reagent for aggregating lipoproteins other than HDL further contains a nonionic surfactant that does not solubilize the aggregated lipoproteins.

(14) The method according to claim (12) or (13), wherein the reagent for aggregating lipoproteins other than HDL is a reagent comprising heparin or a salt thereof, phosphotungstic acid or a salt thereof, dextran sulfuric acid or a salt thereof, polyethylene glycol, sulfonated cyclodextrin or a salt thereof, sulfonated oligosaccharide or a salt thereof, or a mixture thereof and a divalent metal salt.

(15) The method according to claim (6) or (11), wherein the CH enzymes used in the first reaction of cholesterol are chemically modified enzymes and the CH enzymes used in the second reaction of cholesterol are enzymes that are not chemically modified.

(16) The method according to any one of claims (10) through (15), wherein the reagent enabling the CH enzymes to

act on cholesterol in all lipoproteins is a reagent containing a lipoprotein solubilizing surfactant.

(17) A reagent for determining LDL cholesterol comprising CH enzymes and a reagent enabling the CH enzymes to act only on LDL cholesterol.

(18) The reagent for determining LDL cholesterol according to claim (17), wherein the reagent enabling the CH enzymes to act only on LDL cholesterol is a reagent containing at least a polyoxyethylene derivative and a polyoxyethylene-polyoxypropylene copolymer.

(19) The reagent according to claim (18), wherein the polyoxyethylene derivative is a polyoxyethylene alkylaryl ether.

(20) The reagent according to claim (18) or (19), wherein the polyoxyethylene-polyoxypropylene copolymer is a surfactant represented by general formula (I):

$$HO-(C_2H_4O)_a-(C_3H_6O)_b-(C_2H_4O)_c-H \qquad (I)$$

(wherein a, b and c, which may be the same or different, each represents an integer of 1 to 200).

(21) A reagent kit for the fractional determination of HDL cholesterol and LDL cholesterol comprising a first reagent and a second reagent, said first reagent comprising a reagent for aggregating lipoproteins other than HDL lipoprotein and a reagent containing CH enzymes, and said second reagent comprising a reagent enabling CH enzymes to act only on LDL cholesterol.

(22) The reagent kit according to claim (21), wherein the reagent enabling CH enzymes to act only on LDL cholesterol is a reagent containing a polyoxyethylene derivative and a polyoxyethylene-polyoxypropylene copolymer.

(23) The reagent kit according to claim (21), wherein the polyoxyethylene derivative is a polyoxyethylene alkylaryl ether.

(24) The reagent kit according to claim (21) or (22), wherein the polyoxyethylene-polyoxypropylene copolymer is a surfactant represented by general formula (I):

$$HO-(C_2H_4O)_a-(C_3H_6O)_b-(C_2H_4O)_c-H \qquad (I)$$

(wherein a, b and c, which may be the same or different, each represents an integer of 1 to 200).

(25) A reagent kit for the fractional determination of HDL cholesterol and total cholesterol comprising a first reagent and a second reagent, said first reagent comprising a reagent for aggregating lipoproteins other than HDL lipoprotein and a reagent containing CH enzymes, and said second reagent comprising a reagent enabling CH enzymes to act on cholesterol in all lipoproteins.

(26) The reagent kit according to claim (25), wherein the reagent enabling CH enzymes to act on cholesterol in all lipoproteins further contains a lipoprotein solubilizing surfactant.

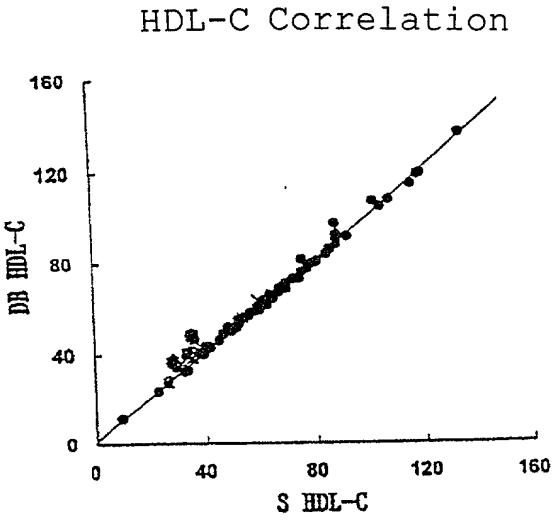
(27) The reagent kit according to any one of claims (21) through (26), wherein the reagent for aggregating lipoproteins other than HDL lipoprotein is a reagent comprising heparin or a salt thereof, phosphotungstic acid or a salt thereof, dextran sulfuric acid or a salt thereof, polyethylene glycol, sulfonated cyclodextrin or a salt thereof, sulfonated oligosaccharide or a salt thereof, or a mixture thereof and a divalent metal salt.

ABSTRACT

The present invention provides a method for the quantitative determination of cholesterol in low density lipoproteins and a reagent kit for use therein. The present invention also provides a method for continuous fractional determination of cholesterol in high density lipoproteins and cholesterol in low density lipoproteins and a reagent kit for use therein, as well as a method for continuous fractional determination of cholesterol in high density lipoproteins and total cholesterol and a reagent kit for use therein.

***

Fig. 1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DB HDL-C
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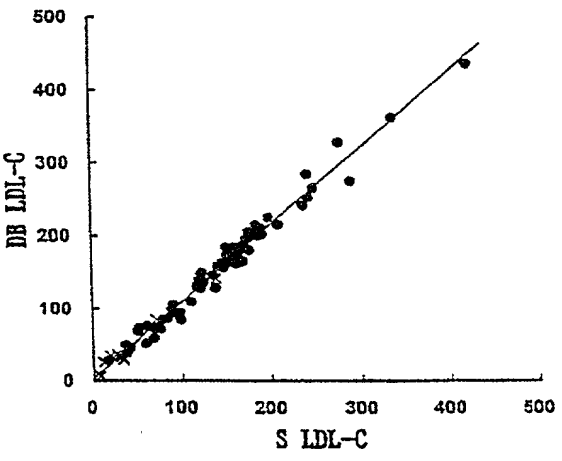
40
80
120
160
S HDL-C

Fig. 2


LDL-C Correlation
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200
100
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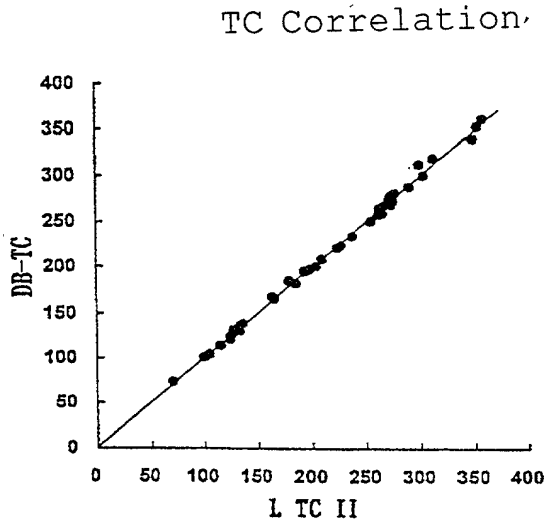
DB LDL-C
0
100
200
300
400
500
S LDL-C

Fig. 3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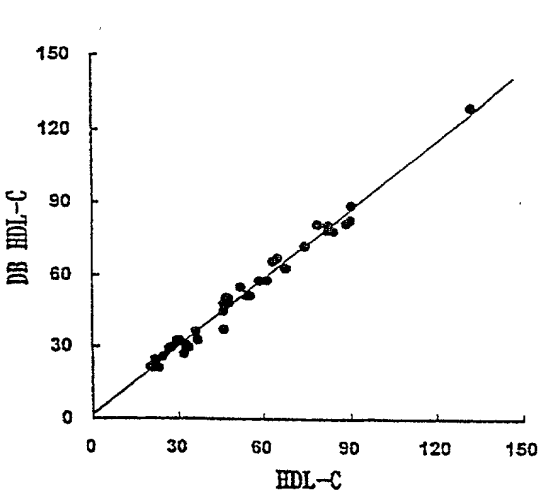
100
150
200
250
300
350
400

Fig. 4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DB HDL-C
HDL-C
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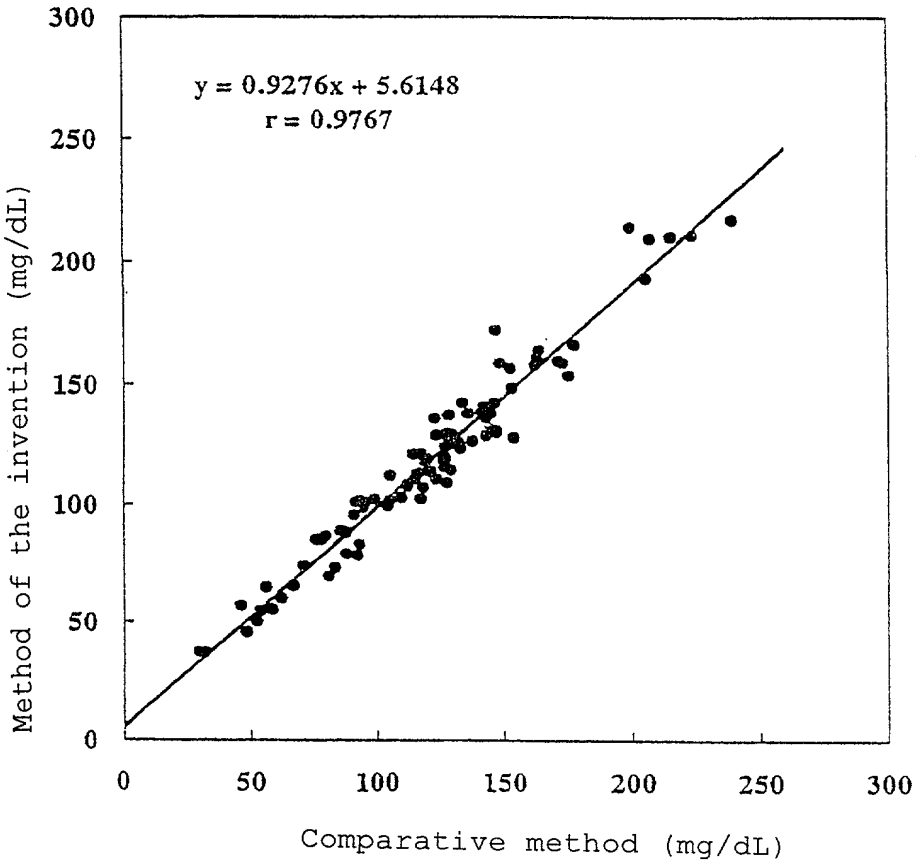
30
60
90
120
150

Fig. 5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r = 0.9767
Method of the invention (mg/dL)
Comparative method (mg/dL)
0
50
100
150
200
250
300

COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT COOPERATION TREATY APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHOD FOR FRACTIONAL DETERMINATION OF CHOLESTEROL IN LIPOPROTEINS AND A REAGENT THEREFOR, the specification of which was filed as PCT International Application No. PCT/JP99/04128 on 30/07/99 and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Country	Application No.	Filed (Day/Mo./Yr.)	Priority Claimed (Yes/No)
JAPAN	264367/98	18/09/98	YES

I hereby appoint the practitioners associated with the firm and Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to the address associated with that Customer Number:

FITZPATRICK, CELLA, HARPER & SCINTO
Customer Number: 05514

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

Full Name of Sole or First Inventor Hiroyuki Sugiuchi

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